

# FAST ELECTRON TRANSFER PROCESSES IN CYTOCHROME C AND RELATED METALLOPROTEINS

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**ABSTRACT** Various free radicals formed on pulse radiolysis of aqueous solutions have been used to investigate the mechanisms of reduction of cytochrome(III) *c* by inter- and intramolecular electron transfer. The rapid formation of free radicals ( $t < 1 \mu\text{s}$ ) and their high reactivity with cytochrome ( $k \approx 10^8 - 5 \times 10^{10} \text{M}^{-1}\text{s}^{-1}$ ) make such studies feasible. Reduction of cytochrome by free radicals is monitored by optical methods. Fast optical changes in the 1-500- $\mu\text{s}$  region correspond to reduction of the iron center; whereas the slower changes in the 10-500-ms region are attributed to postreduction conformational changes. It has been concluded that the reduction path is mediated through the crevice and that no reduction intermediates are being formed.

## INTRODUCTION

Kinetic studies of electron transfer processes in heme proteins (1-3) have been one of the major approaches in determining the mechanistic behavior and subtle structural features of these large electron carriers found in living systems. These kinetic measurements made *in situ* and in isolated systems are continually improving our understanding of the electron transport systems in general and the mechanisms of energy conversion in particular.

The search for ever faster kinetic techniques brought about the development in the early 60s (4) of pulse radiolysis, which is one of the most convenient techniques in the study of fast electron transfer processes and in some applications has a time resolution approaching picoseconds (5).

The kinetic studies presented here of some free radical reactions with cytochrome *c*, cyt(III)-*c*, in aqueous solutions represent an attempt to ascertain the exact paths an electron takes toward its focal point, Fe(III). It has already been demonstrated (6) that the reactions of some free radicals with cyt(III)-*c* can lead to quantitative reduction, yielding an unperturbed cyt(II)-*c*. It has also been stated (6) that reduction of heme proteins by free radicals under controlled radiolytic conditions may have certain advantages over classic reducing agents, which may involve as intermediates either H atoms (e.g.,  $\text{H}_2/\text{Pt}$ ) or other free radicals derived from two electron reductants (e.g., dithionite, ascorbate).

## METHODS

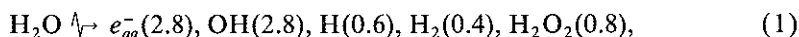
Primary water free radicals were generated by irradiating aqueous solutions with either Co-60  $\gamma$ -rays, referred to as steady-state radiolysis, at dose rates 1.8 krad/min or by high energy (2 MeV) electron beams of short duration (50 ns), referred to as pulse radiolysis, at dose rates 1–2 krad/pulse. In the pulse experiments, a Febetron 705 was used as a generator of pulsed electron beams in conjunction with a kinetic spectrophotometric detection system with time resolution of 0.5  $\mu$ s (7). Standard pulse radiolysis procedures were used to obtain absorption spectra of the transients and the products and to determine the corresponding rate constants.

High purity Millipore-filtered water (Millipore Corp., Bedford, Mass.) and chemicals were used in preparing solutions. Sigma cyt(III)-c, type VI (Sigma Chemical Co., St. Louis, Mo.), from horse heart prepared in the absence of trichloroacetic acid (a strong electron and free radical scavenger) was used without further purification. All solutions were 1 mM in phosphate buffer, and the pH was adjusted with KOH. Purified carboxymethylated cytochrome *c* (kindly supplied by Dr. R. E. Eakin), with both methionine moieties converted into  $>\text{S}^+-\text{CH}_2\text{CO}_2^-$  was used (8). Whale myoglobin(III) was used without purification. Physiological integrity of both cyt(II)-c and myoglobin(II) was fully demonstrated.

## RESULTS

### *Generation of Free Radicals*

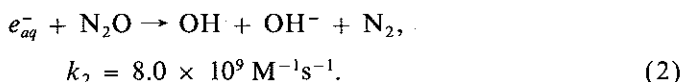
Free radicals were generated by reaction of added solutes with the primary water free radicals, which in turn were formed initially either on steady-state or pulse radiolysis of aqueous solutions (4):



where *G* values (number of species formed/100 eV of energy absorbed) in parentheses, strictly speaking, refer to dilute solutions and may vary to a certain extent depending on the nature and concentration of the dissolved material.

Considering the probable connection between the protein free radicals presumably formed in the cytochrome and the redox processes, we generated model peptide free radicals for investigation, the choice being made on the basis of high reactivities of the cytochrome components for  $e_{aq}^-$  or OH. Related model radicals were also employed. All the free radicals used and the compounds from which they were generated are shown in Table I. The concentrations of the solutes were chosen on the basis of particular reaction rate constants (9, 10).

The reactions of OH radicals were always studied in the presence of  $\text{N}_2\text{O}$  because the hydrated electron, a strong reductant ( $E^\circ = -2.8$  V), readily converts to OH radical, a strong oxidant ( $E^\circ = +2$  V):



Electron adducts of imidazole and the peptide bond are easy to generate because of their high reactivity with the hydrated electron (9), e.g.,

TABLE I  
REDUCTION RATE CONSTANTS FOR CYT(III)-c BY FREE RADICALS IN  
AQUEOUS SOLUTIONS AT 20°C

Solute	{S} mM	pH	Radical	$k, \text{M}^{-1}\text{s}^{-1}$
<i>t</i> -Butanol	10	7.0	$e_{aq}^-$	$5.5 \times 10^{10}$ (ref. 15)
<i>t</i> -Butanol*	10	7.0	$(\text{CH}_3)_2\text{CH}_2\text{COH}$	No reaction
Formate*	10	7.0	$\cdot \text{CO}_2^-$	$1.3 \times 10^9 \ddagger$
Gly anhydride*	10	6.8	$\text{CH}_2\text{CONHCHCONH}$	$< 10^7$
Gly anhydride	10	6.8	$-\dot{\text{C}}\text{OHNH}-$	$8.0 \times 10^8$
Pentaerythritol*	100	6.8	$(\text{CH}_2\text{OH})_3\text{C}\dot{\text{C}}\text{HOH}$	$< 10^6$
	100	9.8	$(\text{CH}_2\text{OH})_3\text{C}\dot{\text{C}}\text{HOH}$	$1.6 \times 10^8$
Benzoate*	10	6.8	$\cdot \text{C}_6\text{H}_5(\text{OH})\text{CO}_2^-$	
Benzoate	10	6.8	$\text{C}_6\text{H}_5\text{COOH}^-$	$1.8 \times 10^9$
Imidazole*	3	7.0	$\cdot \text{Im}-\text{OH}$	$7.5 \times 10^8$
Imidazole§	3	6.3	$\text{Im}^- (\text{H}^+) \cdot$	$< 10^7$
His-His*	1	7.1	$\cdot \text{His}-\text{OH}$	$2.1 \times 10^8$
His-His§	10	6-7	$\text{His}^- (\text{H}^+) \cdot$	$< 10^7$
Met*	10	6.8	$\text{Met}-\text{OH}  $	$1.0 \times 10^9$
Phe*	10	6.8	$\text{Phe}-\text{OH}$	
Acetophenone	10	7.0	$\text{C}_6\text{H}_5\dot{\text{C}}\text{OHCH}_3$	$8.0 \times 10^8$
<i>p</i> -Nitroacetophenone	2	7.0	$^- \text{O}_2\text{N}-\text{C}_6\text{H}_4\text{COCH}_3$	$1.2 \times 10^8$

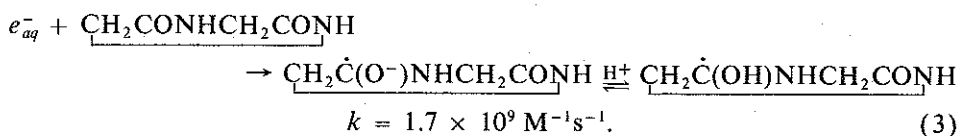
\*In the presence of 1 atm  $\text{N}_2\text{O}$  to convert  $e_{aq}^-$  into OH radicals.

‡ Ref. 16 indicates a decrease in rate with increasing formate concentration.

§ In the presence of 100 mM pentaerythritol to eliminate OH radicals—the resulting pentaerythritol radical does not show any reaction under pulse conditions and pH < 7.

|| Poor reductant even under steady-state conditions,  $k$  probably  $< 1 \text{ M}^{-1}\text{s}^{-1}$ .

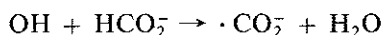
¶ Or free radicals derived from it.



The corresponding electron adducts of Phe, Tyr, and Trp were not amenable to investigation because of their much lower  $k$  values ( $\sim 10^8 \text{ M}^{-1}\text{s}^{-1}$ ) relative to cyt(III)-c. The electron adduct of benzoate (II) was used as a substitute for Phe.

When electron adducts were generated, OH radicals were removed with solutes giving rise to nonreducing radicals, e.g., *t*-butanol radicals or to pentaerythritol radicals, which are unreactive under pulsed conditions in the time frame for observation.

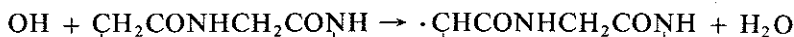
One of the strongest reducing radicals ( $E^\circ = -2 \text{ V}$ ), excluding  $e_{aq}^-$  and H atoms, is  $\cdot \text{CO}_2^-$  (12). It is usually produced via



$$k = 2.0 \times 10^9 \text{ M}^{-1}\text{s}^{-1} \quad (10)$$

No reaction of  $\cdot\text{CO}_2^-$  was observed with any of the protein components. The consequences of this observation for the mechanisms of reduction of the cytochrome are discussed below.

The  $\alpha$ -peptide radicals are also reducing agents, though not as good as electron adducts of the peptide bond or  $\cdot\text{CO}_2^-$ . They can be conveniently produced via



$$k = 1.2 \times 10^9 \text{ M}^{-1}\text{s}^{-1} \quad (5)$$

Besides the abstraction reaction, i.e., reactions 4 and 5, OH readily adds (10) to the benzene ring (Phe, Tyr), to heterocyclic systems (His, Trp) and to sulfur (Met, Cys). The resulting free radicals are shown in Table I.

In addition to the mechanism for generating a free radical, one has to consider its acid-base properties (13), because the electron transfer processes depend on the state of protonation of the donor as well as of the acceptor.

#### Reaction Rate Constants

The  $k$  values for reduction were derived primarily from the growth of the  $\alpha$ -band at 550 nm. When possible, the decay of absorbance of the reducing radical was also followed. Unfortunately, the absorption changes produced upon reduction of the cytochrome in most cases masked the absorption bands of the free radicals. The absorption changes were followed over at least two half-lives. The resulting rate constants are shown in Table I.

The  $k$  values have been found to be pH-dependent at  $\text{pH} > 7$ . In Fig. 1 the pH dependence for  $\text{O}\text{NO}_2^-$  and  $(\text{CH}_3)_2\dot{\text{C}}\text{OH}$  radicals is shown. In solutions of nitrobenzene, the OH radical gives  $\text{HO}-\text{O}\text{NO}_2$ , which is unreactive with  $\text{cyt(III)-c}$ , i.e.,  $k < 10^7 \text{ M}^{-1}\text{s}^{-1}$ , allowing the  $\text{O}\text{NO}_2^-$  reaction to be followed. The solutions of isopropanol were saturated with  $\text{N}_2\text{O}$ , hence the  $(\text{CH}_3)_2\dot{\text{C}}\text{OH}$  radicals were generated exclusively.

The pentaerythritol radical  $(\text{CH}_2\text{OH})_3\dot{\text{C}}\text{CHOH}$  is formed by abstraction of hydrogen by OH (14). This radical quantitatively reduces the cytochrome under steady-state conditions (6) but fails to do so under pulsed conditions; at  $\text{pH} < 7$  and because of high concentrations, these radicals preferentially recombine before having a chance to react with the cytochrome. An increase of pH above 7 makes the cytochrome more reactive, and  $k = 1.6 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$  at pH 9.8 was measured (14). The increase in the extent of cytochrome reduction with increasing pH is shown in Fig. 2. Above pH 10 the extent of reduction begins to fall off, presumably as a result of some other changes in the cytochrome.

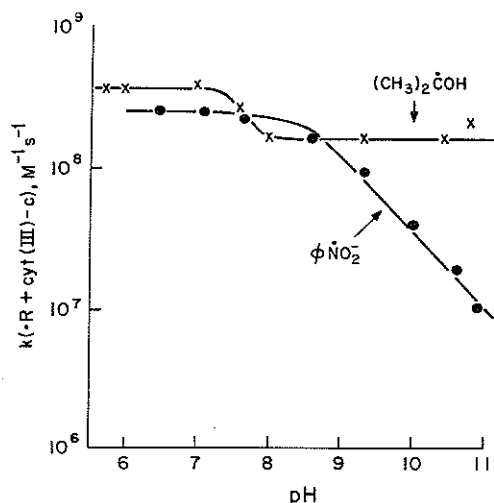


FIGURE 1

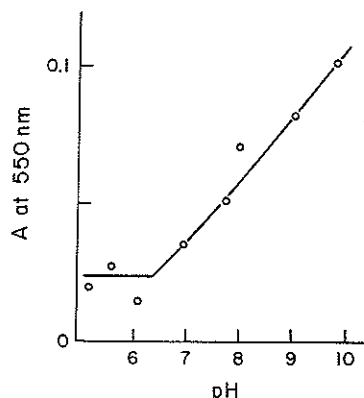


FIGURE 2

FIGURE 1 The pH dependence of reduction rate constants for cyt(III)-c by  $\phi\text{NO}_2^-$  and  $(\text{CH}_3)_2\dot{\text{C}}\text{OH}$  radicals. Conditions: —O—,  $2 \times 10^{-5}\text{M}$  cyt(III)-c,  $5 \times 10^{-3}\text{M}$  nitrobenzene,  $10^{-3}\text{M}$  phosphate buffer; —X—,  $2 \times 10^{-5}\text{M}$  cyt(III)-c,  $5 \times 10^{-2}\text{M}$  isopropanol,  $10^{-3}$  phosphate buffer, 1 atm  $\text{N}_2\text{O}$ . Dose/pulse = 0.5 krad.

FIGURE 2 The pH dependence of the change in absorption at 550 nm corresponding to the reduction of cyt(III)-c by the pentaerythritol radical,  $(\text{CH}_2\text{OH})_3\dot{\text{C}}\text{CHOH}$ . Conditions:  $2 \times 10^{-5}\text{M}$  cyt(III)-c,  $2 \times 10^{-2}\text{M}$  pentaerythritol,  $10^{-3}$  phosphate buffer, 1 atm  $\text{N}_2\text{O}$ . Dose/pulse = 1.6 krad.

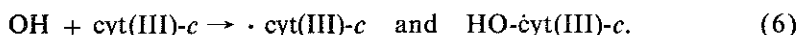
#### Reduction by the Hydrated Electron

The hydrated electron is one of the strongest reducing agents, and much attention has been paid to its reactions (4). The rate constant of  $e_{aq}^-$  reaction with cyt(III)-c is extremely high,  $k = 5.5 \times 10^{10}\text{M}^{-1}\text{s}^{-1}$  (15), and it has been suggested that the full development of cyt(II)-c absorption is not concomitant with electron decay (15). The delayed development has a  $t_{1/2} = 7\text{ }\mu\text{s}$ , attributed to intramolecular electron transfer from the initial reaction site to the Fe center (15). These observations were made in the presence of 0.5 M *t*-butanol, but such a first-order change is not observed either when formate (16) or when 0.1 M pentaerythritol in this study were used as OH scavengers.

A 100% reduction yield by  $e_{aq}^-$  under steady-state conditions (6) and under pulsed conditions can be observed. However, the buildup of cyt(II)-c makes the yield vs. dose plot nonlinear because of the rather strong reactivity of cyt(II)-c toward  $e_{aq}^-$ ,  $k = 2.0 \times 10^{10}\text{M}^{-1}\text{s}^{-1}$ .

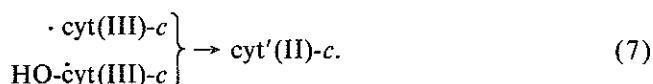
#### Reaction of the OH Radical

The OH radical has been observed to reduce cyt(III)-c (6). The following multistep sequence is suggested. First, OH abstracts H or adds to aromatic and sulfur-containing residues.



The  $k_6 = 2.7 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$  value was derived from competition studies.

Some of the resulting free radicals on this protein of the cytochrome appear to reduce Fe(III) intramolecularly,



This reduction was followed at 550 nm, and two distinct first-order rate constants were derived,  $k'_1 = 2 \times 10^5 \text{ s}^{-1}$  and  $k''_1 = 3 \times 10^4 \text{ s}^{-1}$ . Although the rates were relatively independent of the batch of the cytochrome, the extent of reduction ranged from 25 to 55%. Because of this apparent irreproducibility, consideration of the OH-induced reduction will be postponed. From Table I it is clear that some OH-induced protein radicals could very efficiently reduce the Fe(III) center.

#### Conformational Changes

Reduction of the cytochrome by most free radicals, as indicated by the growth of the  $\alpha$ -band, takes place in the 20–500- $\mu\text{s}$  time regime in these experiments. This change is then followed by a slower absorption change with  $t_{1/2} = 0.1 \text{ ms}$ , which was attributed to conformational changes (16).

In this study the extent of the slow change was found to be variable (0–50% of the total  $\Delta A$ ) and dependent on the batch of cytochrome. It cannot be correlated to any free radical reaction, and it is most likely affected by the conformation of the cytochrome. No meaningful correlation can be drawn at present.

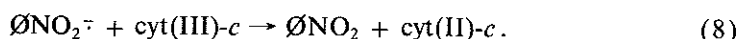
#### DISCUSSION

Most reducing free radicals ( $E' < 0 \text{ V}$ ) reduce cyt(III)-c with rate constants about  $10^8 - 2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ . The exceptions (14), such as  $\cdot \text{O}_2^-$  ( $k = 1.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  at pH < 7) and flavine mononucleotide semiquinone radical ( $k = 1.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$  at pH 7), fall into a category of free radicals with redox potentials close to that of the cytochrome ( $E' = +0.25 \text{ V}$ ).

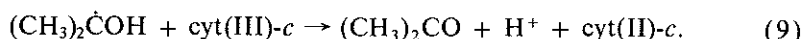
In addition to energetics, steric factors play a substantial role, as in the case of the pentaerythritol radical,  $(\text{CH}_2\text{OH})_3\text{C}\dot{\text{C}}\text{HOH}$ , which has a neopentane structure. The relatively low rate constant for reduction by this radical at pH < 7 ( $k < 10^6 \text{ M}^{-1} \text{ s}^{-1}$ ) cannot be rationalized on purely energetic grounds because that radical reacts with hemin(III)-c ( $E' \sim -0.2 \text{ V}$ ) fast,  $k = 3.0 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$  (17). Though reduction rate at pH < 7 is low, it increases at pH > 7 ( $k = 1.6 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$  at pH 10), suggesting that the reduction path is through the crevice, which widens at higher pHs. The absence of initial reduction at external sites on the protein is apparent. This absence is consistent with the fact that the pentaerythritol radical does not react with either the peptide bond (e.g. glycine anhydride) or aromatic amino acids (Phe, His, etc.). The  $\cdot \text{CO}_2^-$  radical, one of the strongest reducing radicals (12), also appears to be unreactive toward the

peptide bond and the aromatic residue, indicating that operation of the so-called "Winfield mechanism" (18) would be highly improbable.

Most of the free radicals reduce the Fe center directly, e.g.,

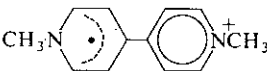
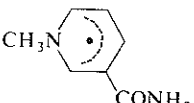


For protonated free radicals the electron transfer is associated with a release of a proton, e.g.,



Despite the observations with the pentaerythritol radical, conformational changes induced by the increased pH (19–21) usually decrease the reduction rate constants as is found for classic reductants. These changes in  $k$  (see Fig. 1) fall into a similar pH range as the reported  $\text{pK}_a = 8.9$  to  $9.3$  (22) for cytochrome conformers. The change that occurs at about pH 7.5 for the  $(\text{CH}_3)_2\dot{\text{C}}\text{OH}$  radical may be the result of denaturing effect of the isopropanol from which the radical is derived. A drop in the  $k$  value for this radical was also observed at pH < 7 for solutions 2 M in isopropanol (14). The effect on  $k$  is therefore a composite one and is dependent on  $\Delta E^\circ$ , steric factors, and the influence of the medium.

TABLE II  
COMPARISON OF REACTION RATE CONSTANTS FOR SOME FREE RADICALS WITH HEMIN C, WHALE MYOGLOBIN, HORSE CYTOCHROME C AND CARBOXY-METHYLATED CYTOCHROME C IN AQUEOUS SOLUTIONS AT pH = 7 AND 20°C

Free radical	$k, \text{M}^{-1} \text{s}^{-1*}$			
	Heme(III)-c†	Mb(III)	cyt(III)-c	(cm) <sub>2</sub> cyt(III)-c§
$e_{aq}^-$	$2.1 \times 10^{10}$	$2.5 \times 10^{10}$	$5.5 \times 10^{10} \parallel$	$4.4 \times 10^{10}$
$\cdot \text{CO}_2^- \P$	$1.3 \times 10^9$	$2.0 \times 10^9$	$1.3 \times 10^9$	$1.4 \times 10^8$
$\text{HOCCO}_2^- \ddagger \ddagger$	$1.0 \times 10^8$	$3.5 \times 10^7$	$1.7 \times 10^8$	$2.8 \times 10^7$
$\text{HOCHCO}_2^-$	$1.0 \times 10^8$	$3.5 \times 10^7$	$1.7 \times 10^8$	$2.8 \times 10^7$
$\phi \text{COOH}^\cdot$	$2.1 \times 10^9$	$1.8 \times 10^9$	$1.8 \times 10^9$	$1.4 \times 10^9$
	** $1.6 \times 10^9$	$2.3 \times 10^8$	$3.6 \times 10^8$	$5.2 \times 10^7$
	** $2.1 \times 10^9$	$8.7 \times 10^8$	$1.4 \times 10^9$	$1.1 \times 10^9$

\*All  $k$  values  $\pm 20\%$ . Mb, myoglobin.

† From ref. 17.

§ M. G. Simic. Submitted for publication.

|| From ref. 15.

¶  $10^{-2}$  M formate.

\*\*  $10^{-1}$  M formate.

‡‡  $10^{-2}$  tartrate.

An effect on free radical reduction kinetics can also be observed in chemically modified cytochromes. In carboxymethylated cyt(III)-c, Met 80 is modified and detached from Fe. The rate constants for reduction by  $\cdot\text{CO}_2^-$ , tartrate, and viologen radicals are consequently reduced by a factor of 6. Replacement of Met 80 sulfur ligand by an alternative protein ligand at pH > 7 (23) and the role of Met 80 in redox processes have already been recognized (24). On the other hand,  $k$  values for aromatic donors with low redox potentials such as electron adducts of benzoate, acetophenone, and 1-methyl nicotinamide are  $\sim 10^9 \text{ M}^{-1} \text{ s}^{-1}$  for the heme compounds. The high  $k$  values for the reduction of cytochrome by aromatic electron donors suggest a mechanism involving  $\pi - \pi$  interactions of the donors, the aromatic residues on the cytochrome and the porphyrin ring. Identical reduction rate constants of  $k = 1.8 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$  for the reaction of the benzoate electron adduct with both the cytochrome and myoglobin, in spite of their totally different configurations, suggest that other interactions can also occur. A  $\pi - \pi$  interaction between the donor and the porphyrin ring may play an important role as indicated by the equivalence of  $k$  values for the benzoate electron adduct reduction of cytochrome, carboxymethylated cytochrome, and myoglobin (see Table II).

Direct and stoichiometric reduction of the Fe in the cytochrome by  $e_{aq}^-$  that does not involve mediation by a longer-lived intermediate (although an intermediate with  $t_{1/2} < 0.15 \mu\text{s}$  could be involved and not detected; 16) is surprising in view of a rather high reactivity of  $e_{aq}^-$  with many of the protein components. This can perhaps be rationalized, considering the effect of charge distribution (high positive charge around the cleft would attract  $e_{aq}^-$ ) and the reluctance of  $e_{aq}^-$  to enter hydrophobic regions of the protein.

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## DISCUSSION

CZERLINSKI: The increase of the reduction of ferricytochrome c by the pentaerythritol radical (Fig. 2) is certainly interesting. You indicate in the text that this reduction decreases at pH 11. This effect is probably due to the second protonic dissociation described in the cited reference of Czerlinski and Dar (21). Your Fig. 1 points to similar deviations at pH 11. Do you have any new information on this point?

SIMIC: Yes. Referring to Fig. 2, the pentaerythritol radical has a neopentane structure and it has difficulties in reducing cytochrome(III)-c to cytochrome(II)-c. The pentaerythritol radical has absolutely no difficulty in reducing hemin-c, which has a redox potential of  $-0.2$  V, much lower than that of cytochrome-c, which is  $E^{01} = +0.250$  V. Hence there are no energetic considerations. Only the size and the structure of the radical is preventing it from reducing cytochrome(II)-c. Now, you are quite right about the high pH effect. I have not shown points beyond pH 10; I just didn't put them on. The  $k$ -values went considerably above pH 10, so something has been happening to cytochrome-c and also to the pentaerythritol radical in this pH region. Namely, it is deprotonating and is in the anionic form. The anionic form is an even better reductant than the protonated form, so the state should not stop it from reducing cytochrome-c. On the other hand, the increase in reduction above pH 7 (Fig. 2) is too far from the  $pK$  value of the radical, which is about 10.4, to be accounted for. Therefore the pH changes involving cytochrome-c here are something you have talked about in your paper

(21). You did also talk about the  $pK$  value around 7, I think, in another paper (Czerlinski, 1973). Right?

CZERLINSKI: The electron transfer-linked  $pK_H$  is near 9. One may wonder how this  $pK_H$  extends its effects all the way to  $pH = 7$ . I have another remark regarding the action of the pentaerythritol radical. Could the electron in the pentaerythritol radical be much more localized than in the others? The chemical structure seems to point in this direction. The electron of the pentaerythritol radical may need to get into close contact with iron(III) and cannot directly interact with the porphyrin system:

SIMIC: Yes, I fully believe in what you said. The thesis of the paper is that the delocalization of the electron, promoted by the aromaticity of the donor, substantially aids the electron transfer.

MAUZERALL: A simple explanation for the slow reaction of the pentaerythritol radical is possible in terms of electron tunneling. It is not so much a question of electron delocalization as it is of distance. In this radical the odd electron on the carbon may be held sufficiently further from the cytochrome by the  $-\text{CH}_2\text{OH}$  groups to slow the electron transfer below that of a radical without such groups. The decrease in rate would be fit by a reasonable tunneling parameter of  $0.5 \text{ \AA}$ .

SIMIC: That's right. The cleft is hydrophobic in nature and these hydroxy derivatives are of course extremely hydrophilic and they might have problems in penetrating to a distance where it could tunnel. But we have not observed tunneling.

AUSTIN: Is it possible to measure the reaction rate of something like oxygen with the hemes in your experiments and get attachment rates?

SIMIC: Yes, you can. In the reaction rate of cytochrome(II)-*c* with oxygen, cytochrome(II)-*c* is extremely stable and does not react with oxygen. However, carboxymethylated cytochrome, which means binding  $-\text{CH}_2\text{CO}_2^-$  to the sulfur of Met-80, results in the release of Met-80 from the iron, so that the iron is left with only five ligands instead of six. The redox potential is considerably reduced and you can measure the rate of oxygen with carboxymethylated cytochrome(II)-*c*. On the other hand, you can also use part of cytochrome-*c*, namely hemin(III)-*c*. The Fe(III) form can be rapidly reduced ( $1-10 \mu\text{s}$ ) with an electron donor. You can then study the reactions of the reduced form with oxygen. This method is widely used in pulse radiolysis. For instance, we find that hemin(II)-*c* reacts with  $\text{O}_2$  with  $k = 4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ . This is quite a fast rate constant, but not quite diffusion controlled.

KLAPPER: I have an answer to Dr. Austin's question on ligand binding to heme proteins previously reduced by the hydrated electron. Two papers have appeared recently (Ilan et al., 1978; Ho et al., 1978) on the reactions of CO and  $\text{O}_2$  to singly reduced hemoglobin. The results from the two laboratories are comparable, though not identical. The rate constants for the binding of  $\text{O}_2$  or CO, in the presence and absence of inositol hexaphosphate, onto hemoglobin with one iron reduced and the remaining three oxidized, are similar to those obtained by other techniques for the binding of the first  $\text{O}_2$  or CO onto the fully reduced protein.

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